

# THE SITE OF RIBONUCLEIC ACID SYNTHESIS IN THE ISOLATED NUCLEUS\*

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There is still controversy concerning the primary site of RNA synthesis in the cell.<sup>1</sup> When tissues are radioautographed after administration of radioactive inorganic phosphorus or radioactive RNA<sup>2</sup> precursors such as tritiated uridine or cytidine, it is commonly observed that radioactivity appears in the nucleus first and in the cytoplasm later. It has furthermore been shown that isolated nuclei of both animal<sup>3</sup> and plant<sup>4</sup> material are able to synthesize RNA *in vitro* but that enucleated cytoplasm is unable to carry on such synthesis.<sup>5</sup> Although the problem of whether or not RNA is synthesized in both nucleus and cytoplasm is not completely understood,<sup>6-8</sup> there is increasing cytological evidence that a substantial portion of the cytoplasmic RNA is synthesized in the nucleus<sup>9-17</sup> and subsequently migrates to the cytoplasm.

The site of RNA synthesis in the nucleus, however, is variously proposed to be the nucleolus,<sup>13, 16, 18</sup> the chromatin,<sup>19-21</sup> or both.<sup>22, 23</sup> It has been suggested that cytoplasmic RNA is derived from either the nucleolus<sup>13, 16, 18</sup> or from the chromatin after passage through the nucleolus<sup>19, 20</sup> or from both.<sup>13</sup> The autoradiographic data heretofore obtained are inconclusive on these points. Autoradiographs of nuclei labeled with radioactive RNA precursors are only rigorously interpretable in the case of isolated nuclei or nucleoli because of the background otherwise created by radioactive cytoplasm or by chromatin closely associated with the nucleolus. In the present work the more direct approach of the physical fractionation of previously labeled, isolated nuclei has been used. Large quantities of nuclei have been isolated, incubated under suitable conditions with labeled precursors of RNA, the subnuclear components then separated from one another, and the amounts of radioactivity contained in the RNA of chromatin, nucleoli, and other nuclear components estimated directly. It has in this way been possible to arrive at reasonably firm conclusions concerning the site of RNA synthesis in the nucleus.

**Materials and Methods.**—*Plant tissues:* Pea seedlings, *Pisum sativum*, were grown in vermiculite in the dark for 4 days at 25°C. From these seedlings, apical tips, 1.0–2.0 cm in length, were cut, freed of vermiculite, and then washed with 0.5% clorox for 3 min. These stem sections were then used for isolation of nuclei.

**Chemicals:** Cytidine-H<sup>3</sup>, specific activity: 4,900 mc per mM, used as RNA precursor and obtained from New England Nuclear Corporation, Boston, was added to the incubation medium in a final concentration of 25  $\mu$ c per ml. Phosphocreatine kinase was prepared from rabbit muscle according to the procedure described by Kuby *et al.*<sup>24</sup> Penicillin powder, specific activity 1,000 units per mg was used as manufactured by Chas. Pfizer & Co., Inc., New York. Other chemicals were of reagent grade.

**Isolation and incubation of nuclei:** Nuclei were isolated from pea seedlings by a new method which consists in passing the plant tissue through a set of counter-rotating rollers with simultaneous addition of a sucrose-CaCl<sub>2</sub> medium; sucrose, 0.25 *M*; CaCl<sub>2</sub>, 0.003 *M*; tris buffer, 0.006 *M*, pH 7.2.<sup>25</sup> We have constructed a semiautomatic machine to perform this operation. The plant tissue is carried on nylon mesh, first chopped into 1 mm sections, and the segments are then

passed through the two rollers. The homogenate is collected and the thus liberated intact nuclei quickly purified by filtration through a double layer of miracloth and subsequent centrifugation at  $350 \times g$  for 10 min. All of the operations are carried out at  $4^{\circ}\text{C}$ . The purity of the nuclear fractions is routinely tested under the light microscope both with the specimens stained with iodine and with the ones stained with acetocarmine. The bulk of the nuclear contaminants are found to be starch particles, and very few particles are found that are both acetocarmine-negative and iodine-negative. The nuclear preparations are completely free of any intact cells.

The isolated pea nuclei were then incubated in a medium (Table 1) containing cytidine- $\text{H}^3$  as

TABLE 1  
INCUBATION MEDIUM FOR CYTIDINE- $\text{H}^3$  INCORPORATION\*

Sucrose	$25 \times 10^{-2} M$
ATP	$10^{-4} M$
GTP	$10^{-4} M$
UTP	$10^{-4} M$
Phosphocreatine	$5 \times 10^{-3} M$
Phosphocreatine kinase 100 $\gamma$ /ml	
Tris buffer	$25 \times 10^{-3} M$
$\text{CaCl}_2$	$3 \times 10^{-3} M$
$\text{MgCl}_2$	$5 \times 10^{-3} M$
Cytidine- $\text{H}^3$ (25 $\mu\text{c}$ )	$5 \times 10^{-6} M$
Final vol.	1 ml
pH 7.2	

\* 0.35 mg of penicillin is added per ml of incubation medium to control bacterial activity.

radioactive RNA precursor at  $35^{\circ}\text{C}$ . Penicillin powder 0.35 mg per ml of incubation medium was added to control bacterial activity. About 6 to 8 mg of nuclear protein was used for each sample for incubation. Uptake of cytidine- $\text{H}^3$  was stopped by rapid chilling and dilution of the radioactive cytidine by a large excess of non-radioactive CTP.

*Isolation of nucleoli, chromatin particles, and other subnuclear components:* To locate the RNA-synthesizing function in the nucleus it has been necessary to separate the subnuclear components. The principle of separation and isolation used has been adapted from that of Johnston *et al.*<sup>26</sup> and has been further extensively developed for our purposes.<sup>27</sup> Rupture of nuclei is accomplished by removal of calcium from the nuclear suspension as the calcium-citrate complex. The nuclei are then ground in an Omni-Mixer at 40 volts for 3 to 4 min. The thus disintegrated nuclear materials are next separated by fractional centrifugation in concentrated sucrose solution. The sucrose solution in which homogenization of nuclei is to be carried out is adjusted to specific gravity of 1.31. After the grinding, the homogenates are diluted with 18% by volume of water and then subjected to fractionation. The most completely nucleolar fraction was obtained by centrifuging for 20 min at 11 to 14 thousand rpm in the Spinco swinging bucket rotor No. 25. After all of the nucleoli were spun down, the homogenate was further 5 times diluted ( $\text{KCl}$ , 10 mM;  $\text{MgCl}_2$ , 2 mM; tris buffer, 6 mM, pH 7.2) and then centrifuged at 20,000 rpm for 15 min in the No. 30 angular rotor. This sediments the chromatin fraction. Further centrifugation at 30,000 rpm for 4 hr resulted in sedimenting of the ribosomal fractions.

*Determination of DNA, RNA, protein, and radioactivity:* The pellets of the various fractions were treated in 3%  $\text{HClO}_4$  for 2 hr at  $0^{\circ}\text{C}$  and were then washed essentially according to the procedure of Ogur and Rosen.<sup>28</sup> This consists in washing once with 70% ethanol, once with 70% ethanol-0.1%  $\text{HClO}_4$ , once with 70% ethanol, twice with ethanol-ether (3:1), and finally with 2%  $\text{HClO}_4$  (all at  $2-4^{\circ}\text{C}$ ). The RNA in the washed precipitate was then hydrolyzed in 1 N  $\text{HClO}_4$  for 60 hr at  $0^{\circ}\text{C}$ . RNA was determined by optical density of the perchloric hydrolysates, using the Cary Spectrophotometer. DNA was extracted from the residue by heating in 0.5 N  $\text{HClO}_4$  in a water bath for 20 min at  $70^{\circ}\text{C}$  and this extraction was repeated once. The combined extracts were then used for DNA determination by optical density at 268  $\mu$ . Protein of the residue was determined by the Biuret method according to the procedure of Gornall *et al.*<sup>29</sup> Aliquots of the 1 N  $\text{HClO}_4$  extract were taken for determination of radioactivity in the RNA sample. For counting, 0.1 ml of the solution was pipetted into a bottle containing 10 ml of the following mixture:<sup>30</sup> 700 ml of toluene, 300 ml of absolute ethanol, 4 gm of 2,5-diphenyloxazole, and 100 mg of 1,4-bis-2-

(5-phenyloxazolyl)-benzene. The sample was then counted in a Packard liquid scintillation spectrometer. The counts were corrected for self-absorption of the 1 *N* HClO<sub>4</sub> solution.

**Results.**—*The site of incorporation of cytidine-H<sup>3</sup> into RNA:* Nuclei isolated as described above actively incorporate several metabolites, including uridine-H<sup>3</sup> and cytidine-H<sup>3</sup>, into RNA<sup>25</sup> and amino acids into protein.<sup>31</sup> As will be described elsewhere,<sup>25</sup> the incorporation of cytidine into RNA is almost wholly dependent upon the presence of the other three nucleoside triphosphates and is increased by the addition of energy-regenerating system. For the experiment of Figure 1,

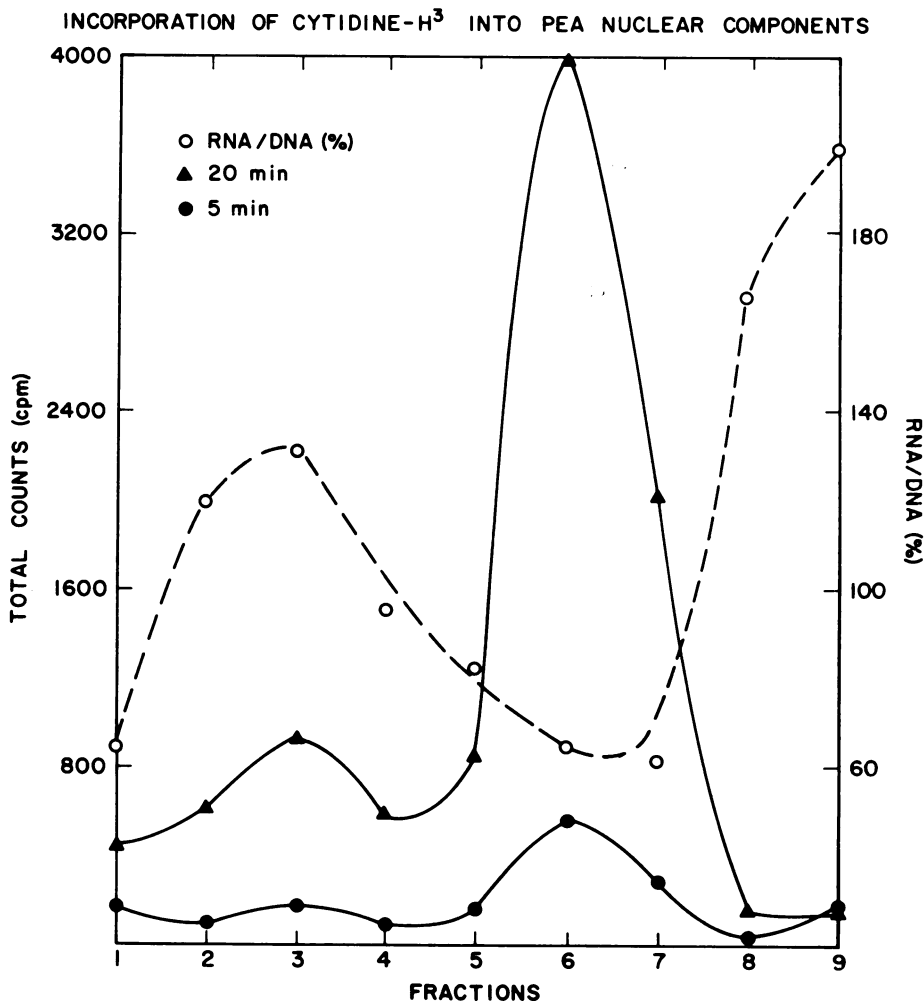


FIG. 1.—Incorporation of cytidine-H<sup>3</sup> into RNA of nuclear components. The disintegrated nuclear materials are fractionated by successive centrifugations in sucrose solution of specific gravity of 1.26. The pellets of fractions 1 to 6 were collected successively from the supernatant of the previous fraction at rpms of 6,000, 11,000, 14,000, 17,000, 20,000, and 25,000 in the Spinco swinging bucket rotor No. 25. Fraction 1 consists principally of unbroken nuclei and starch granules. Fractions 2 and 3 are principally nucleoli. Fractions 4 and 5 consist of nucleoli mixed with chromatin particles. The supernatant of Fraction 6 was diluted 5-fold and then centrifuged at 20,000 rpm for 15 min in the No. 30 angular rotor to obtain Fraction 7. Fractions 6 and 7 are free of intact nucleoli and are referred to chromatin fraction. Further centrifugation of the supernatant of Fraction 7 at 30,000 rpm for 4 hr results in sedimenting of Fraction 8 which corresponds to the ribosomal fraction. Fraction 9 is simply the supernatant of Fraction 8.

isolated nuclei were allowed to incorporate cytidine- $H^3$  into RNA (medium described in Table 1), the reaction stopped after 5 min by chilling, the reaction mixture diluted with a large amount of non-radioactive cytidine, and the nuclei then ground and fractionated into subnuclear fractions. It is clear from Figure 1 that labeling appears principally in two fractions, namely fractions 3 and 6. Fraction 3 which exhibits a moderately high RNA-to-DNA ratio is the principle nucleolar fraction as determined with the light microscope. The other peak of labeling coincides with fraction 6, which has a low RNA-to-DNA ratio and which represents chromatin material free of nucleoli. The labeling of the chromatin fraction in a 5-min period is about 3 to 4 times greater than that of the nucleolar fraction. If the incubation period is prolonged to 20 min however, the labeling in the two fractions becomes approximately equal, and the subsequent rates of incorporation into the two fractions remain approximately equal.

Fraction 8 (Fig. 1) is that which includes the ribosomal material (Ts'o *et al.*<sup>32</sup>) while Fraction 9 includes all supernatant (smaller than ribosomal) materials. Neither ribosomal nor supernatant fraction gain any large amount of label even after 20 min of incubation.

When the pattern of nuclear labeling is plotted in terms of specific activity, cpm/unit RNA, rather than in terms of total activity per fraction as in Figure 1, only one peak is observable, namely that of the chromatin fraction. The nucleolar fraction by contrast exhibits rather low specific activity even after 20 min of incubation.

*The dilution of the radioactive RNA precursor pool with unlabeled CTP:* In these experiments, isolated nuclei were incubated in cytidine- $H^3$ -containing medium for a certain period of time, an excess of unlabeled CTP (1,000 times as much as the amount of cytidine- $H^3$  used) added to quench incorporation of label, and the nuclei then allowed to continue to synthesize RNA for various periods. When the non-radioactive CTP was added, the incubation mixture was placed in ice and left for 5 min to equilibrate the CTP with radioactive RNA precursor pool, after which time the incubation mixture was returned to 35°C. Total activity incorporated into nuclear RNA in this type of experiment increases over the first 10 min after addition of the non-radioactive CTP. The kinetics of this increase (Fig. 2), which are fairly consistent for many nuclear preparations and all time intervals, consist of an initial suspension of incorporation (*ca.* 5 min) followed by approximately 5 min of further synthesis. After this period, however, appearance of label in RNA ceased entirely as should be expected. With these reservations then, the fate of the previously synthesized RNA can be followed. The characteristics of the distribution of label among subnuclear fractions after quenching are shown in Figures 3 and 4. Incorporation of cytidine- $H^3$  into the RNA of chromatin continues as described above for 10 min beyond the instant of quenching. After this time, however, the amount of activity in chromatin decreases or at least fails to increase further. Both the nucleoli and the mixed chromatin-nucleoli fractions, on the other hand, initially gain activity slowly, although at an increasing rate, up to the time of quenching. After dilution of the radioactive RNA precursor pool with non-radioactive CTP, the activity in the RNA of these two fractions, unlike that of chromatin, does not increase merely for 10 min but on the contrary increases rapidly for up to 30 min.

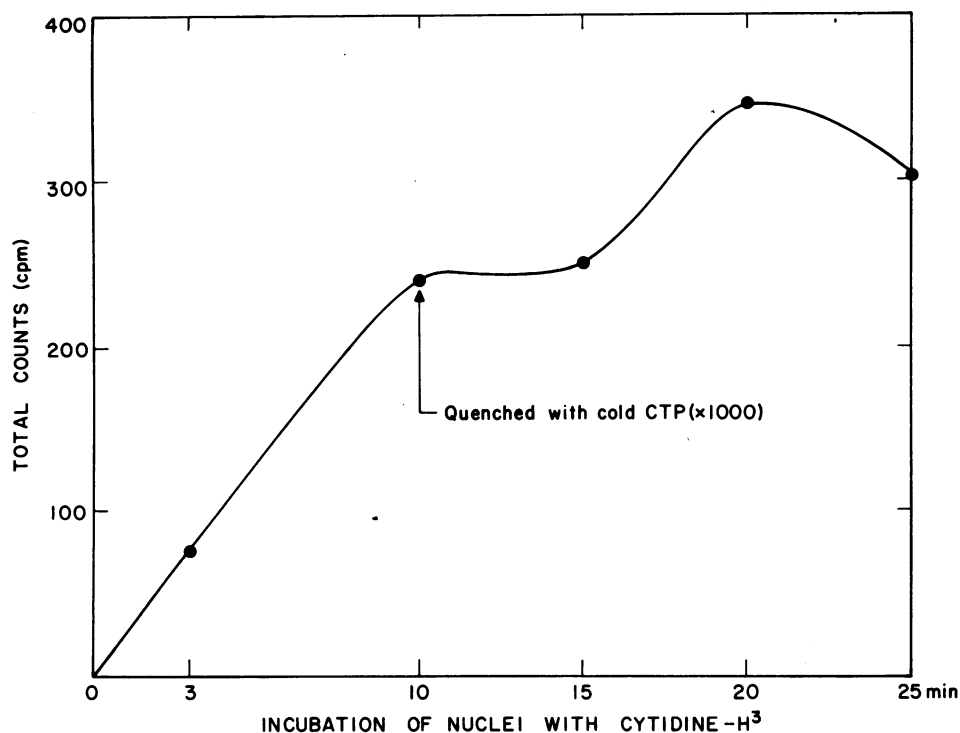


FIG. 2.—Time course of cytidine- $H^3$  incorporation into RNA of isolated nuclei. Nuclei were incubated at  $35^\circ C$  in the medium of Table 1 for 10 min and the incubation mixtures then diluted with an excess of non-radioactive CTP (1,000 times as much as the amount of cytidine- $H^3$  used). After dilution of the medium with non-radioactive CTP, the incubation mixtures were left in ice for 5 min and then returned to  $35^\circ C$  for further incubation. 0.35 mg of penicillin powder was added. Total volume, 1.0 ml.

But little activity appears in the ribosomal fraction during the first 30 min of incubation. After this time, however, some labeling does appear in this fraction.

In Figure 4, the data of Figure 3 are transformed to specific activity. It is clear that the specific activity of the nucleolar RNA, initially less than that of the chromatin RNA, rises steadily after quenching and ultimately becomes equal to or greater than the latter. It is clear then, both from the data on total activity incorporated and from that on specific activity, that the chromatin fraction initially incorporates cytidine- $H^3$  into RNA at a faster rate than does the nucleolar fraction but that the activity of chromatin levels off earlier than does that of nucleoli after dilution of the radioactive RNA precursor pool by non-radioactive CTP.

*Discussion.*—The above results show that during short-time incubations, uptake of cytidine- $H^3$  into RNA takes place in two regions of the nucleus, namely the chromatin and the nucleolus. The chromatin exhibits a much greater initial rate of incorporation than does the nucleolus, but the amount of labeled RNA in the nucleolus does increase with time after a prolonged lag period. Several interpretations of this observation would in principle be possible. Thus, it might be supposed that RNA is synthesized essentially only in the chromatin portion of the nucleus, that it is associated with chromatin for a short time, and that it then migrates to the nucleolus. One might further suppose that the newly formed RNA remains

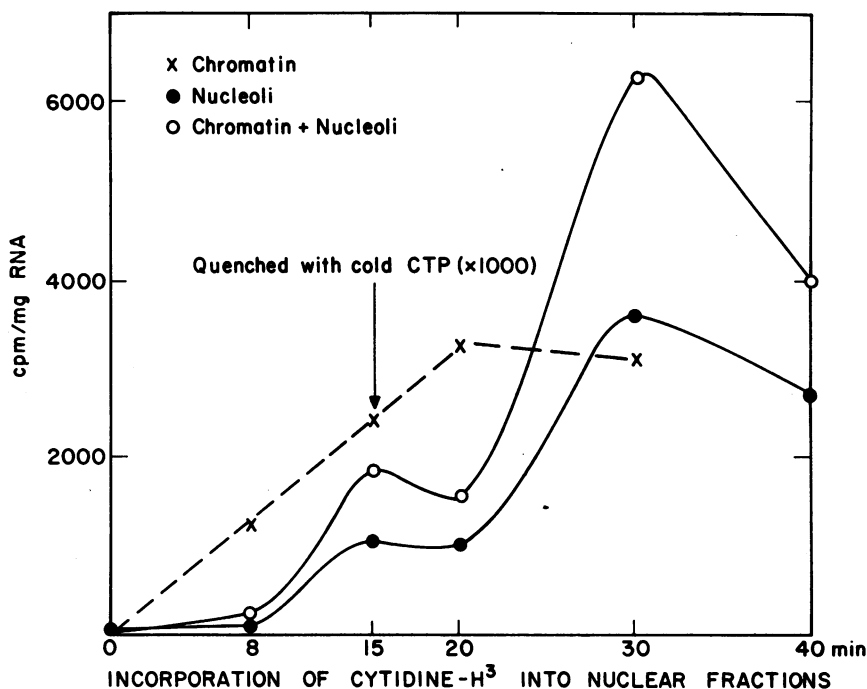


FIG. 3.—Time course of cytidine- $H^3$  incorporation into RNA of nuclear components. Incubation conditions as for Figure 2, except that dilution of the incubation mixtures with non-radioactive CTP was after 15 min of incubation.

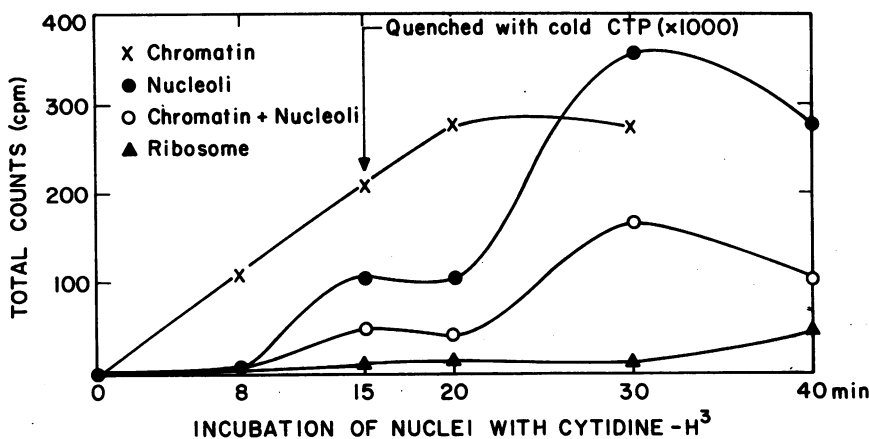


FIG. 4.—Time course of cytidine- $H^3$  incorporation into nuclear components. Incubation conditions as for Figure 3.

in the nucleolus for a relatively long time and then moves out to the cytoplasm. A second possible interpretation would be that chromatin and nucleolus are equally capable of incorporating cytidine- $H^3$  into RNA and that the labeling characteristic depends on either the pool sizes of unlabeled precursors in each component or the total RNA concentration of each component. This second possibility is, however, eliminated at once by the fact that quenching of the incorporation of label into

RNA by non-labeled CTP decreases appearance of label in chromatin but increases appearance of labeled RNA in nucleoli.

Previous workers have disagreed on the initial site of RNA synthesis in the nucleus. Several investigators who have used the technique of counting of the number of grains per unit area over each nuclear structure in autoradiograms and after incubation in labeled precursor for a relatively long time (1 hr or longer) have observed that nucleoli become the most heavily labeled structure in the nucleus and have suggested therefore that RNA is synthesized in the nucleolus, from whence it moves to the cytoplasm. Most of the autoradiographic data has been expressed in terms of concentration of label or number of grains per unit area over each structure. Different conclusions would be reached if one were to express the same data as total label incorporated into each nuclear component. By counting total grains in autoradiograms over each nuclear component and by using much shorter times of incubation (4 to 5 min), it has been found that a small but significant amount of labeled RNA appears in the chromosomal portion of interphase nuclei and that during these short incubations nucleoli incorporate but little radioactivity.<sup>19, 20, 22</sup> Woods<sup>22</sup> in his autoradiographic study found that steady-state incorporation of labeled RNA in the chromatin is reached 14 min after the application of label to *Vicia* tissue. During further incubation, the amount of labeled RNA in the nucleolus rises steadily to reach a level considerably higher than that in the chromatin, a final steady-state distribution between chromatin and nucleolus being reached approximately 1.5 hr after the beginning of treatment. Goldstein and Micou,<sup>19</sup> using similar techniques, have found that the amount of RNA which appears in the nucleolus during a 5- to 10-min interval is of the order of magnitude which would be expected if all of the labeled RNA of the nucleolus were derived from the chromosomal RNA, and without independent RNA synthesis in the nucleolus. It may be concluded therefore that the results of the present preparative and chemical approach support the previous autoradiographic evidence and provide strong support for the hypothesis that the chromatin is the primary site of synthesis of a large portion of the cellular RNA.

The question of whether or not the simultaneous decline of labeling in chromatin RNA and the gain of labeling in nucleolar RNA after quenching is by direct transfer of large RNA molecules to the nucleolus or by degradation to small molecules followed by resynthesis is an important one. If there were any independent incorporation of labeled cytidine in the nucleolus or any reincorporation of labeled cytidine after degradation of labeled chromosomal RNA, this should be stopped by the dilution of the labeled precursor pool with non-radioactive CTP. Under these circumstances, nucleolar incorporation should be expected to cease immediately, just as is the case with incorporation into the chromatin fraction. In fact, however, the nucleolar fraction gains radioactive RNA in spite of such dilution. It can therefore be concluded that the label incorporated into nucleolar RNA comes from a source other than the cytidine- $H^3$  pool. The probable explanation seems to us to be that it comes from the chromatin as a large molecule.

The present data are by no means complete enough to exclude the possibility that there are, at least in part, distinct and different RNAs in the nucleolus and in the chromatin or that they may have their own independent rates of synthesis and degradation. Many workers have shown that nuclear RNA may be com-

posed of at least two different fractions<sup>4, 22, 23, 33-35</sup> and there is no reason to suppose that these two fractions are themselves homogeneous. If there are many RNA species and fractions in the nucleus, each may have its own characteristic kinetics of labeling in experiments such as those presented above.

The characteristic time course of labeling of the chromatin and nucleolar fractions found in the present study suggest however that the RNA is actually synthesized on the chromatin with which it remains associated for a time which is short in comparison to the time which it spends associated with the nucleolus. It may be understood on this basis why the appearance of labeled RNA in the nucleolus exhibits an early lag but later rises to high levels. The RNA, assembled in close association with the DNA of the chromatin, would appear according to the present view to be transferred to the nucleolus, where, over a substantial period of time, it is modified or processed for its ultimate cellular function.

*Summary.*—Nuclei isolated from pea tissue by a new method, which consists in passing the tissues through a set of counter-rotating rollers, have been used to study the site of RNA synthesis in the nucleus. Nuclei, previously incubated in a mixture including tritium-labeled cytidine, were fractionated into subnuclear components. It has been found that incorporation of cytidine into RNA takes place in two regions of the nucleus, namely in the chromatin and in the nucleolus. Uptake of label by chromatin is, however, initially much more rapid than uptake by other subnuclear components. Appearance of label in nucleolar RNA is characterized by a prolonged lag period.

In further experiments, nuclei were allowed to incorporate label into RNA for a short period, and the radioactive RNA precursor pool was then diluted with a large excess of unlabeled CTP. Under these circumstances, label disappears from the RNA of chromatin and appears in the RNA of nucleoli. It is concluded that RNA is first synthesized on the chromatin and is then transferred to the nucleolus.

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<sup>2</sup> Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, GTP, UTP, and CTP, 5'-triphosphates of adenosine, guanosine, uridine, and cytidine; Tris, tris (hydroxymethyl) aminomethane; cpm, counts per minute; rpm, revolutions per minute.

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## ULTRAVIOLET ACTION SPECTRA OF ORDERED AND DISORDERED DNA\*

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It has been postulated that ordered and disordered DNA have different ultraviolet action spectra, although the absorption spectra are similar in shape,<sup>1</sup> and that the action spectrum criterion may be used to diagnose organization of DNA in biological systems.<sup>2</sup> There are three lines of experimental evidence supporting this hypothesis.

1. The single-stranded DNA bacteriophage  $\Phi$ X174 (ref. 3), when assayed for loss of plaque formation, has an ultraviolet action spectrum with a minimum at a longer wavelength than that of the double-stranded DNA bacteriophage T2. The action spectrum of  $\Phi$ X174 changes from pH 2–12 like the changes in the absorption spectrum of the sum of the DNA pyrimidines,<sup>4</sup> which are known to be more ultraviolet-sensitive than the DNA purines.<sup>5</sup> However, the absorption spectrum of  $\Phi$ X174 reflects the sum of the absorption of both purines and pyrimidines.

2. During intracellular development of T2 the action spectrum for loss of plaque formation approaches the shape of the  $\Phi$ X174 spectrum.<sup>2</sup> The interpretation of this shift as a change from a two- to a one-stranded state of the viral DNA is supported by the appearance of ability to fix complement in intracellular T4 DNA,<sup>6</sup> this serological activity being associated with heat-denatured but not native DNA.